

Functional Homologies between Avian and Human Alphaherpesvirus VP22 Proteins in Cell-to-Cell Spreading as Revealed by a New *cis*-Complementation Assay[†]

C. Blondeau, D. Marc, K. Courvoisier, J.-F. Vautherot, and C. Denesvre*

Laboratoire de Virologie Moléculaire, INRA, UR1282, Infectiologie Animale et Santé Publique, IASP, Nouzilly F-37380, France

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VP22, encoded by the *UL49* gene of Marek's disease virus (MDV), is indispensable for virus cell-to-cell spreading. We show herein that MDV *UL49* can be functionally replaced with avian and human viral orthologs. Replacement of MDV VP22 with that of avian gallid herpesvirus 3 or herpesvirus of turkey, whose residue identity with MDV is close to 60%, resulted in 73 and 131% changes in viral spreading, respectively. In contrast, VP22 replacement with human herpes simplex virus type 1 resulted in 14% plaque formation. Therefore, heterologous avian and human VP22 proteins share sufficient structural homology to support MDV cell-to-cell spreading, albeit with different efficiencies.

UL49 gene-encoded VP22 is specific to alphaherpesviruses. This 249- to 304-amino-acid protein is a major constituent of the virus tegument layer. *UL49* functional requirements seem to vary from one virus to another and depending on the host cell. In pseudorabies virus, herpes simplex virus type 1 (HSV-1), and bovine herpesvirus type 1 (BoHV-1), *UL49* appears to be not essential for viral replication in cell culture (2, 6–8, 11). In HSV-1 and BoHV-1, however, deletion of *UL49* impairs virus replication, especially in MDBK cells (7, 11). In HSV-1, the absence of VP22 is associated with (i) a decrease in the incorporation of several HSV-1 proteins into virions, (ii) a toxic effect probably due to the uncontrolled RNase activity

encoded by *UL41*, and (iii) a decrease in extracellular particle accumulation (6, 7, 15). *UL49* has been shown to be absolutely necessary for the replication of Marek's disease virus (MDV) and varicella-zoster virus in cell culture (5, 16). Despite these differences between alphaherpesviruses, previous amino acid alignments of VP22 unveiled the presence of a conserved central domain suggestive of a conserved function (4, 12, 13). Herein, we tested whether other alphaherpesvirus *UL49* genes, either from the same *Mardivirus* genus or from a more phylogenetically distant human virus, could replace MDV's *UL49* gene by *cis* complementation in an MDV genomic background.

Genus	Species	N	C	AA Identity (entire VP22)	Sequences used (access n°)
Mardivirus	MDV	1	96 173 249	100 %	YP_001033978
Mardivirus	GaHV3	1	94 171 241	72 %	NP_066881
Mardivirus	HVT	1	136 213 283	64 %	AAG45787
Varicellovirus	VZV	1	158 235 302	54 %	NP_040132
Simplexvirus	HSV1	1	178 255 301	41 %	CAA32299

FIG. 1. Schematic representation of several avian and human VP22 proteins and their percent homologies with MDV VP22. The MDV VP22 polypeptide sequence was aligned pairwise with each ortholog by using Bestfit (GCG package; Accelrys). Grey boxes represent the conserved core of VP22. The percent homologies (amino acid [AA] identities with MDV VP22) were calculated for (i) the conserved core region (written in the boxes) and for (ii) the entire VP22 protein, except for varicella-zoster virus (VZV) and HSV-1, which exhibited low levels of similarity outside the conserved area (see the supplemental material). NA, not appropriate.

* Corresponding author. Mailing address: Laboratoire de Virologie Moléculaire, INRA, UR1282, Infectiologie Animale et Santé Publique, IASP, Nouzilly F-37380, France. Phone: 33-2-47427619. Fax: 33-2-47427774. E-mail: denesvre@tours.inra.fr.

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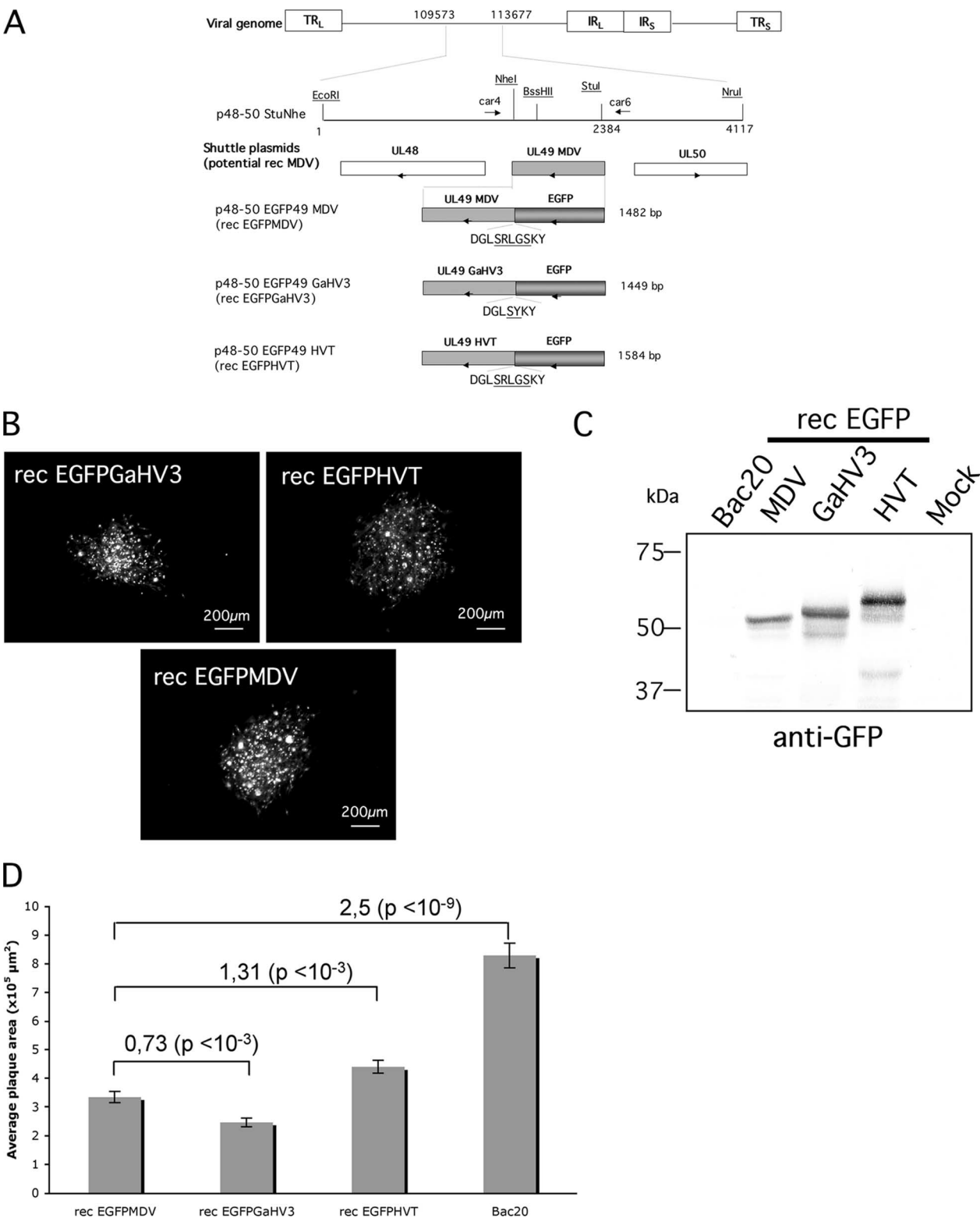


FIG. 2. Construction and cell-to-cell spreading of the recombinant MDVs containing EGFP-tagged *UL49* genes derived from three *Mardivirus* species. (A) Schematic representation of the three shuttle plasmids used for homologous recombination by cotransfection in CESC with the Bac20delUL49 DNA bacmid. (B) Picture of a plaque (one for each virus) with EGFP fluorescence. (C) Analysis of EGFPV22 protein expression by immunoblotting revealed with a rabbit anti-GFP antibody. Mock, noninfected cells. (D) Plaques size comparison after plaque staining. Fifty plaques were analyzed with the cell observer system (Zeiss, Göttingen, Germany) on the red channel, and plaque size was measured with the Axiovision software. Statistical analysis (analysis of variance) showed a significant difference among the three viruses ($P < 10^{-3}$). The ratio between the average plaque area for each virus and that of recEGFPMDV is given, as well as the P values (Student's t test).

TABLE 1. Characteristics of the MDV genome *cis* complemented with several avian and human UL49 open reading frames

ORF ^a inserted at MDV UL49 position	Homologous recombination in CESC with Bac20ΔUL49		Homologous recombination in <i>E. coli</i> with Bac20	
	Progeny	% Cell-to-cell spreading efficiency ^b	Progeny	% Cell-to-cell spreading efficiency ^c
None/Kan ^r	No	0	No	0
MDV UL49	Yes	250	Yes	100
MDV EGFPUL49	Yes	100	Yes (NS ^d)	49 (NS)
GaHV-3 EGFPUL49	Yes	73	ND ^e	ND
HVT EGFPUL49	Yes	131	ND	ND
HSV-1 UL49	ND	ND	Yes	14
HSV-1 EGFPUL49	ND	ND	No	0

^a ORF, open reading frame.^b Versus recEGFPM DV.^c Versus Bac20.^d NS, not shown.^e ND, not done.

Efficient *cis* complementation of MDV cell-to-cell spreading with UL49 genes from other mardiviruses. The homologies (percent amino acid identities) between MDV VP22 and its orthologs from the two other *Mardivirus* species, herpesvirus of turkey (HVT) and gallid herpesvirus 3 (GaHV-3), are 56 and 59%, respectively (Bestfit software; Fig. 1). This percent homology was even over 60% in the core region. To assess the ability of the UL49 genes from HVT and GaHV-3 to *cis* complement the MDV genome lacking UL49, we generated recombinant viruses by homologous recombination in chicken embryonic skin cells (CESC). For this purpose, we cotransfected the nonreplicative Bac20delUL49 clone (MDV lacking UL49) with the p48-50 shuttle plasmid containing the UL49 ortholog in its MDV genetic environment as previously described (3). As no antibody was available to detect either HVT or GaHV-3 VP22, both the HVT and GaHV-3 UL49 orthologs were tagged with enhanced green fluorescent protein (EGFP) at the 3' end of the construct (Fig. 2A). After two blind passages of the cotransfected cells on CESC, replicative viruses designated recEGFP HVT and recEGFP GaHV-3 were obtained with both UL49 orthologs (Table 1). Expression of the HVT or the GaHV-3 EGFPVP22 protein was monitored on infected cells by the green fluorescence (Fig. 2B) and by immunoblotting with a rabbit anti-EGFP antibody as previously described (3) (Fig. 2C). The apparent molecular masses, 53 kDa for GaHV-3 and 57 kDa for HVT, closely matched the predicted sizes. Moreover, sequencing of the PCR products obtained with primers car4 and car6 (Fig. 2A shows the position) on extracted viral DNA showed correct recombinations and sequences (not shown). To estimate the functional efficiencies of the HVT and GaHV-3 VP22 proteins, the cell-to-cell spreading of recombinant viruses recEGFP HVT and recEGFP GaHV-3 was compared to that of the previously described virus MDVEGFPVP22 (3), renamed herein recEGFPMDV. To this aim, viral plaques obtained after infection of CESC for 4 days were fixed and stained for MDV antigens VP5, ICP4, and gB as already described (1). Plaque size was determined by measuring 50 plaques after fluorescent red staining and image analysis with the axiovision software (Zeiss, Göttingen, Germany). There were significant differences in plaque size among the three viruses ($P < 10^{-3}$ by analysis of variance after square root transformation) (Fig. 2D), showing

a difference in the efficacy of cell-to-cell spreading. Interestingly, while GaHV-3 VP22 led to a 1.4-fold decrease in plaque size, HVT VP22 increased plaque size by 1.4-fold, as measured in two independent experiments.

Substantial *cis* complementation of MDV cell-to-cell spreading with the divergent human HSV-1 UL49 gene. We next assessed whether avian MDV VP22 could be replaced with VP22 from a distant genus. We chose herein to test HSV-1 VP22 because it is very divergent from MDV VP22 (only 41% homology in the core region) and has been described as not mandatory for HSV-1 cell-to-cell spreading. We constructed two recombinant MDV strains by homologous recombination with the HSV-1 UL49 gene, one with and one without a 5' EGFP tag sequence (EGFPHSV and HSV, respectively). The procedure we used was two-step red recombination in *Escherichia coli* as described by Tischer et al. (17). For the first recombination step, we constructed the p48-50 kana IscE "en passant" plasmids as schematically represented in Fig. 3 (for details, see the supplemental material). The first recombination was obtained after the transformation of EL250 bacteria containing the Bac20 bacmid with either the 2,911-bp or the 3,634-bp XmnI/HpaI restriction fragment from the HSV or EGFPHSV en passant plasmid, respectively. After the second recombination step, the mutant bacmids were verified by sequencing between the XmnI/HpaI restriction sites. The bacmids recHSV and recEGFPHSV were next transfected into CESC by the calcium phosphate method. For the recHSV bacmid, a viral progeny was obtained after one passage, demonstrating successful *cis* complementation. Expression of the heterologous VP22 protein was verified by fluorescence on infected cells and by immunoblotting with AGVO31, an anti-HSV-1 polyclonal antibody (9) (Fig. 3B). The cell-to-cell spreading efficacy of the recHSV virus, evaluated by measuring plaque size in three independent experiments, was reduced by 6.25- to 8.25-fold compared to that of parental Bac20. However, even though the spreading of this virus was limited, it was still able to propagate to neighboring cells, leading to small plaques, in contrast to parental Bac20 lacking UL49 (Bac20delUL49) leading only to single cells expressing late viral antigens (5). For the recHSV strain, the sequence integrity of MDV UL41 was verified to ensure that compensation did not occur elsewhere, as was previously reported for replicative UL49-null HSV-1 (15).

No replicative virus could be obtained after the transfection of the recEGFPHSV bacmid despite several passages. However, an EGFP signal was observed in single cells after transfection, indicating that the fusion protein is well expressed but the signal is lost after a few passages. Moreover, this bacmid was readily rescued after cotransfection into CESC with the p48-50 StuNhe plasmid containing MDV UL49. The rescued virus produced plaques similar in size to those of Bac20 (not shown), showing that the recEGFPHSV replication defect was due not to an unexpected mutation elsewhere in the genome but to the HSV EGFPUL49 gene insertion itself. Therefore, fusing EGFP to the 3' portion of HSV UL49 completely abrogated HSV-1 VP22 residual biological activity in this context.

This study aimed to evaluate whether heterologous UL49 could *cis* complement an MDV UL49-null phenotype. Our

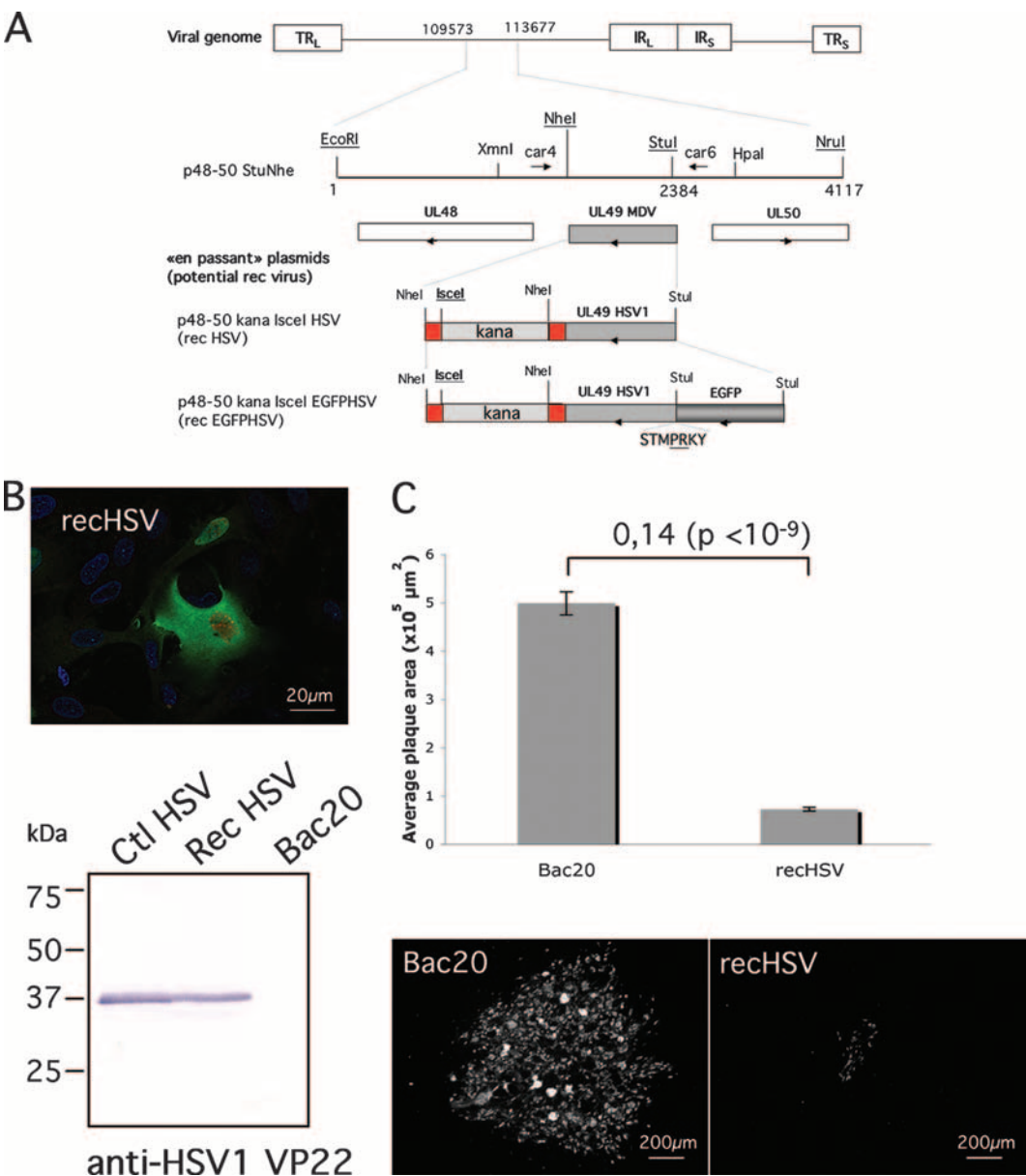


FIG. 3. Construction and cell-to-cell spreading of recombinant MDV containing the HSV-1 *UL49* gene. (A) Schematic representation of the two en passant plasmids constructed and used for the first step of recombination in *E. coli*. (B) Expression of HSV-1 VP22 in recHSV-infected CESC analyzed by fluorescence and immunoblotting. CTLHSV corresponds to CESC transfected with an HSV-1 VP22 expression vector. (C) Plaque size comparisons and plaque pictures viewed on the red channel.

study brings the first evidence for a structural/functional conservation among four VP22 proteins from different avian and human genuses. Although VP22 functional homologies within highly homologous mardiviruses were not surprising, the increased plaque formation observed after the introduction of HVT VP22 points at the potential emergence of more pathogenic MDV strains. These findings are reminiscent of lethal mutations affecting the gB gene in pseudorabies virus that were fully *trans* complemented with BoHV-1 gB, which shares 63% amino acid identity with pseudorabies gB (10). In the present study, the functional complementation of the MDV *UL49*-null phenotype provided by HSV VP22 was more striking because the two proteins are very divergent. This suggests that protein-

protein interactions required for MDV cell-to-cell spreading and involving VP22 may then be partially preserved among VP22 orthologs. A recent study supporting this hypothesis has shown functional proteins interactions between *UL34/UL31* heterologous pairs within the *Betaherpesvirus* subfamily (14). Lastly, the present study confirms that MDV can be a useful and valuable model for studying various aspects of VP22 function, as well as identifying alphaherpesvirus proteins involved in cell-to-cell spreading.

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